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HCV ASSAY

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HCV ASSAY

Cross-Reference to Related Application

This application is related to provisional application no. 60/409,515, filed September 9, 2002, from which priority is claimed under 35 USC §119(e)(1) and which application is incorporated herein by reference in its entirety.

Technical Field

The present invention pertains generally to viral diagnostics. In particular, the invention relates to antigen/antibody/antigen sandwich assays utilizing a first isolated antigen derived from a region of the hepatitis C virus polyprotein and a multiple epitope fusion antigen including multiple epitopes from the HCV polyprotein, which multiple epitopes include one or more epitopes from the same region of the polyprotein as the first antigen, for accurately diagnosing hepatitis C virus infection.

Background Of The Invention

Hepatitis C Virus (HCV) is the principal cause of parenteral non-A, non-B hepatitis (NANBH) which is transmitted largely through blood transfusion and sexual contact. The virus is present in 0.4 to 2.0% of blood donors. Chronic hepatitis develops in about 50% of infections and of these, approximately 20% of infected individuals develop liver cirrhosis which sometimes leads to hepatocellular carcinoma. Accordingly, the study and control of the disease is of medical importance.

HCV was first identified and characterized as a cause of NANBH by Houghten et al. The viral genomic sequence of HCV is known, as are methods for obtaining the

sequence. See, e.g., International Publication Nos. WO 89/04669; WO 90/11089; and WO 90/14436. HCV has a 9.5 kb positive-sense, single-stranded RNA genome and is a member of the Flaviridae family of viruses. At least six distinct, but related genotypes of HCV, based on phylogenetic analyses, have been identified (Simmonds et al., *J. Gen. Virol.* (1993) 74:2391-2399). The virus encodes a single polyprotein having more than 3000 amino acid residues (Choo et al., *Science* (1989) 244:359-362; Choo et al., *Proc. Natl. Acad. Sci. USA* (1991) 88:2451-2455; Han et al., *Proc. Natl. Acad. Sci. USA* (1991) 88:1711-1715). The polyprotein is processed co- and post-translationally into both structural and non-structural (NS) proteins.

In particular, as shown in Figure 1, several proteins are encoded by the HCV genome. The order and nomenclature of the cleavage products of the HCV polyprotein is as follows: NH_2-C-E1-E2-p7-NS2-NS3-NS4a-NS4b-NS5a-NS5b-COOH. Initial cleavage of the polyprotein is catalyzed by host proteases which liberate three structural proteins, the N-terminal nucleocapsid protein (termed "core") and two envelope glycoproteins, AE1" (also known as E) and AE2" (also known as E2/NS1), as well as nonstructural (NS) proteins that contain the viral enzymes. The NS regions are termed NS2, NS3, NS4 and NS5. NS2 is an integral membrane protein with proteolytic activity and, in combination with NS3, cleaves the NS2-NS3 sissle bond which in turn generates the NS3 N-terminus and releases a large polyprotein that includes both serine protease and RNA helicase activities. The NS3 protease serves to process the remaining polyprotein. In these reactions, NS3 liberates an NS3 cofactor (NS4a), two proteins (NS4b and NS5a), and an RNA-dependent RNA polymerase (NS5b). Completion of polyprotein maturation is initiated by autocatalytic cleavage at the NS3-NS4a junction, catalyzed by the NS3 serine protease.

A number of general and specific polypeptides useful as immunological and diagnostic reagents for HCV, derived from the HCV polyprotein, have been described. See, e.g., Houghton et al., European Publication Nos. 318,216 and 388,232; Choo et al., *Science* (1989) 244:359-362; Kuo et al., *Science* (1989) 244:362-364; Houghton et al.,

Hepatology (1991) 14:381-388; Chien et al., *Proc. Natl. Acad. Sci. USA* (1992) 89:10011-10015; Chien et al., *J. Gastroent. Hepatol.* (1993) 8:S33-39; Chien et al., International Publication No. WO 93/00365; Chien, D.Y., International Publication No. WO 94/01778. These publications provide an extensive background on HCV generally, as well as on the manufacture and uses of HCV polypeptide immunological reagents. For brevity, therefore, the disclosure of these publications is incorporated herein by reference.

Sensitive, specific methods for screening and identifying carriers of HCV and HCV-contaminated blood or blood products would provide an important advance in medicine. Post-transfusion hepatitis (PTH) occurs in approximately 10% of transfused patients, and HCV has accounted for up to 90% of these cases. Patient care as well as the prevention and transmission of HCV by blood and blood products or by close personal contact require reliable diagnostic and prognostic tools. Accordingly, several assays have been developed for the serodiagnosis of HCV infection. See, e.g., Choo et al., *Science* (1989) 244:359-362; Kuo et al., *Science* (1989) 244:362-364; Choo et al., *Br. Med. Bull.* (1990) 46:423-441; Ebeling et al., *Lancet* (1990) 335:982-983; van der Poel et al., *Lancet* (1990) 335:558-560; van der Poel et al., *Lancet* (1991) 337:317-319; Chien, D.Y., International Publication No. WO 94/01778; Valenzuela et al., International Publication No. WO 97/44469; and Kashiwakuma et al., U.S. Patent No. 5,871,904.

A significant problem encountered with some serum-based assays is that there is a significant gap between infection and detection of the virus, often exceeding 80 days. This assay gap may create great risk for blood transfusion recipients. To overcome this problem, nucleic acid-based tests (NAT) that detect viral RNA directly, and HCV core antigen tests that assay viral antigen instead of antibody response, have been developed. See, e.g., Kashiwakuma et al., U.S. Patent No. 5,871,904; Beld et al., *Transfusion* (2000) 40:575-579.

However, there remains a need for sensitive, accurate diagnostic and prognostic tools in order to provide adequate patient care as well as to prevent transmission of HCV by blood and blood products or by close personal contact.

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Summary of the Invention

The present invention is based in part, on the finding that the use of a first antigen derived from the HCV polyprotein, in combination with a multiple epitope fusion antigen that includes an epitope from the same region of the HCV polyprotein as the first antigen, provides a sensitive and reliable method for detecting HCV. The assays described herein can detect HCV infection caused by any of the six known genotypes of HCV. In one representative embodiment of the invention, the first antigen includes an NS3/4a conformational epitope and the second antigen is a multiple epitope fusion antigen that includes one or more epitopes from the NS3/4a region. The use of multiple epitope fusion proteins decreases masking problems, improves sensitivity and detects antibodies by providing a greater number of epitopes on a unit area of substrate and improving selectivity. Moreover, the assays described herein can be performed quickly and greater sample volumes can be used without background effects.

Accordingly, in one embodiment, the subject invention is directed to a method of detecting hepatitis C virus (HCV) infection in a biological sample. The method comprises:

(a) providing an immunoassay solid support comprising HCV antigens bound thereto, wherein the HCV antigens consist of one or more isolated antigens from a first region of the HCV polyprotein;

(b) combining a biological sample with the solid support under conditions which allow HCV antibodies, when present in the biological sample, to bind to the one or more HCV antigens;

(c) adding to the solid support from step (b) under complex-forming conditions a detectably labeled HCV multiple epitope fusion antigen (MEFA), wherein the labeled MEFA comprises at least one epitope from the same region of the HCV polyprotein as the one or more isolated antigens, wherein the MEFA binds the bound HCV antibody;

(d) detecting complexes formed between the HCV antibody and the one or more antigens from the first region of the HCV polyprotein and said MEFA, if any, as an indication of HCV infection in the biological sample.

In another embodiment, the invention pertains to a method of detecting HCV infection in a biological sample. The method comprises:

(a) providing an immunoassay solid support comprising HCV antigens bound thereto, wherein the HCV antigens consist of one or more multiple epitope fusion antigens (MEFAs);

(b) combining a biological sample with the solid support under conditions which allow HCV antibodies, when present in the biological sample, to bind to the one or more MEFAs;

(c) adding to the solid support from step (b) under complex-forming conditions a detectably labeled isolated HCV antigen from a region of the HCV polyprotein present in the one or more MEFAs, wherein the isolated antigen binds the bound HCV antibody;

(d) detecting complexes formed between the HCV antibody and the isolated HCV antigen and said MEFA, if any, as an indication of HCV infection in the biological sample.

In still a further embodiment, the invention pertains to a method of detecting HCV infection in a biological sample. The method comprises:

(a) providing an immunoassay solid support comprising HCV antigens bound thereto, wherein the HCV antigens consist of one or more isolated HCV NS3/4a conformational epitopes;

(b) combining a biological sample with the solid support under conditions which allow HCV antibodies, when present in the biological sample, to bind to the one or more NS3/4a epitopes;

(c) adding to the solid support from step (b) under complex-forming conditions a detectably labeled HCV multiple epitope fusion antigen (MEFA), wherein the labeled

MEFA comprises at least one epitope from the HCV NS3/4a region, wherein the MEFA binds the bound HCV antibody;

(d) detecting complexes formed between the HCV antibody and the NS3/4a conformational epitope and the MEFA, if any, as an indication of HCV infection in the biological sample.

In another embodiment, the invention pertains to a method of detecting HCV infection in a biological sample. The method comprises:

(a) providing an immunoassay solid support comprising HCV antigens bound thereto, wherein the HCV antigens consist of one or more multiple epitope fusion antigens (MEFAs) wherein the one or more MEFAs comprise at least one epitope from the HCV NS3/4a region;

(b) combining a biological sample with the solid support under conditions which allow HCV antibodies, when present in the biological sample, to bind to the one or more MEFAs;

(c) adding to the solid support from step (b) under complex-forming conditions a detectably labeled HCV NS3/4a conformational epitope, wherein the NS3/4a conformational epitope binds the bound HCV antibody;

(d) detecting complexes formed between the HCV antibody and the NS3/4a conformational epitope and the MEFA, if any, as an indication of HCV infection in the biological sample.

In the above methods, the NS3/4a conformational epitope and/or the MEFA comprises an epitope from the NS3/4a protease region of the HCV polyprotein and/or an epitope from the NS3/4a helicase region of the HCV polyprotein. In particular embodiments, the NS3/4a conformational epitope comprises the amino acid sequence depicted in Figures 3A-3D (SEQ ID NOS:1 and 2).

In additional embodiments, the MEFA comprises amino acids 1193-1657, numbered relative to the HCV-1 sequence. In yet further embodiments, the MEFA

comprises an epitope from the c33c region of the HCV polyprotein, such as amino acids 1211-1457 and/or amino acids 1192-1457, numbered relative to HCV-1.

In further embodiments, the MEFA comprises an epitope from the 5-1-1 region of the HCV polyprotein, such as amino acids 1689-1735, numbered relative to HCV-1.

In particular embodiments, the MEFA comprises the amino acid sequence depicted in Figures 6A-6F (SEQ ID NOS:3 and 4) or the amino acid sequence depicted in Figures 8A-8F (SEQ ID NOS:5 and 6).

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are set forth herein which describe in more detail certain procedures or compositions, and are therefore incorporated by reference in their entirety.

Brief Description of the Drawings

Figure 1 is a diagrammatic representation of the HCV genome, depicting the various regions of the polyprotein from which the present assay reagents (proteins and antibodies) are derived.

Figure 2 is a schematic drawing of a representative antigen/antibody/antigen sandwich assay under the invention, using MEFA 12.

Figures 3A through 3D (SEQ ID NOS:1 and 2) depict the DNA and corresponding amino acid sequence of a representative NS3/4a conformational antigen for use in the present assays. The amino acids at positions 403 and 404 of Figures 3A through 3D represent substitutions of Pro for Thr, and Ile for Ser, of the native amino acid sequence of HCV-1.

Figure 4 is a diagram of the construction of pd.HCV1a.ns3ns4aPI.

Figure 5 is a diagrammatic representation of MEFA 12.

Figures 6A-6F (SEQ ID NOS:3 and 4) depict the DNA and corresponding amino acid sequence of MEFA 12.

Figure 7 is a diagrammatic representation of MEFA 7.1.

Figures 8A-8F (SEQ ID NOS:5 and 6) depict the DNA and corresponding amino acid sequence of MEFA 7.1.

Figures 9A-9C show representative MEFAs for use with the subject immunoassays. Figure 9A is a diagrammatic representation of MEFA 3. Figure 9B is a diagrammatic representation of MEFA 5. Figure 9C is a diagrammatic representation of MEFA 6.

Detailed Description of the Invention

The practice of the present invention will employ, unless otherwise indicated, conventional methods of chemistry, biochemistry, recombinant DNA techniques and immunology, within the skill of the art. Such techniques are explained fully in the literature. See, e.g., *Fundamental Virology*, 2nd Edition, vol. I & II (B.N. Fields and D.M. Knipe, eds.); *Handbook of Experimental Immunology*, Vols. I-IV (D.M. Weir and C.C. Blackwell eds., Blackwell Scientific Publications); T.E. Creighton, *Proteins: Structures and Molecular Properties* (W.H. Freeman and Company, 1993); A.L. Lehninger, *Biochemistry* (Worth Publishers, Inc., current addition); Sambrook, et al., *Molecular Cloning: A Laboratory Manual* (2nd Edition, 1989); *Methods In Enzymology* (S. Colowick and N. Kaplan eds., Academic Press, Inc.).

All publications, patents and patent applications cited herein, whether *supra* or *infra*, are hereby incorporated by reference in their entirety.

It must be noted that, as used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to "an antigen" includes a mixture of two or more antigens, and the like.

The following amino acid abbreviations are used throughout the text:

Alanine: Ala (A)	Arginine: Arg (R)
Asparagine: Asn (N)	Aspartic acid: Asp (D)
Cysteine: Cys (C)	Glutamine: Gln (Q)
Glutamic acid: Glu (E)	Glycine: Gly (G)
Histidine: His (H)	Isoleucine: Ile (I)
Leucine: Leu (L)	Lysine: Lys (K)
Methionine: Met (M)	Phenylalanine: Phe (F)
Proline: Pro (P)	Serine: Ser (S)
Threonine: Thr (T)	Tryptophan: Trp (W)

Tyrosine: Tyr (Y) Valine: Val (V)

I. Definitions

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

The terms "polypeptide" and "protein" refer to a polymer of amino acid residues and are not limited to a minimum length of the product. Thus, peptides, oligopeptides, dimers, multimers, and the like, are included within the definition. Both full-length proteins and fragments thereof are encompassed by the definition. The terms also include postexpression modifications of the polypeptide, for example, glycosylation, acetylation, phosphorylation and the like. Furthermore, for purposes of the present invention, a "polypeptide" refers to a protein which includes modifications, such as deletions, additions and substitutions (generally conservative in nature), to the native sequence, so long as the protein maintains the desired activity. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutations of hosts which produce the proteins or errors due to PCR amplification.

An HCV polypeptide is a polypeptide, as defined above, derived from the HCV polyprotein. The polypeptide need not be physically derived from HCV, but may be synthetically or recombinantly produced. Moreover, the polypeptide may be derived from any of the various HCV strains and isolates, such as, but not limited to, any of the isolates from strains 1, 2, 3, 4, 5 or 6 of HCV. A number of conserved and variable regions are known between these strains and, in general, the amino acid sequences of epitopes derived from these regions will have a high degree of sequence homology, e.g., amino acid sequence homology of more than 30%, preferably more than 40%, when the two sequences are aligned. Thus, for example, the term "NS3/4a" polypeptide refers to native NS3/4a from any of the various HCV strains, as well as NS3/4a analogs, muteins and immunogenic fragments, as defined further below. The complete genotypes of

many of these strains are known. See, e.g., U.S. Patent No. 6,150,087 and GenBank Accession Nos. AJ238800 and AJ238799.

The terms "analog" and "mutein" refer to biologically active derivatives of the reference molecule, or fragments of such derivatives, that retain desired activity, such as immunoreactivity in the assays described herein. In general, the term "analog" refers to compounds having a native polypeptide sequence and structure with one or more amino acid additions, substitutions (generally conservative in nature) and/or deletions, relative to the native molecule, so long as the modifications do not destroy immunogenic activity. The term "mutein" refers to peptides having one or more peptide mimics ("peptoids"), such as those described in International Publication No. WO 91/04282. Preferably, the analog or mutein has at least the same immunoactivity as the native molecule. Methods for making polypeptide analogs and muteins are known in the art and are described further below.

Particularly preferred analogs include substitutions that are conservative in nature, i.e., those substitutions that take place within a family of amino acids that are related in their side chains. Specifically, amino acids are generally divided into four families: (1) acidic -- aspartate and glutamate; (2) basic -- lysine, arginine, histidine; (3) non-polar -- alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar -- glycine, asparagine, glutamine, cysteine, serine threonine, tyrosine. Phenylalanine, tryptophan, and tyrosine are sometimes classified as aromatic amino acids. For example, it is reasonably predictable that an isolated replacement of leucine with isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar conservative replacement of an amino acid with a structurally related amino acid, will not have a major effect on the biological activity. For example, the polypeptide of interest may include up to about 5-10 conservative or non-conservative amino acid substitutions, or even up to about 15-25 conservative or non-conservative amino acid substitutions, or any integer between 5-25, so long as the desired function of the molecule remains intact. One of skill in the art may readily

determine regions of the molecule of interest that can tolerate change by reference to Hopp/Woods and Kyte-Doolittle plots, well known in the art.

By "fragment" is intended a polypeptide consisting of only a part of the intact full-length polypeptide sequence and structure. The fragment can include a C-terminal deletion and/or an N-terminal deletion of the native polypeptide. An "immunogenic fragment" of a particular HCV protein will generally include at least about 5-10 contiguous amino acid residues of the full-length molecule, preferably at least about 15-25 contiguous amino acid residues of the full-length molecule, and most preferably at least about 20-50 or more contiguous amino acid residues of the full-length molecule, that define an epitope, or any integer between 5 amino acids and the full-length sequence, provided that the fragment in question retains immunoreactivity in the assays described herein. For example, preferred immunogenic fragments for use in the MEFAs, include but are not limited to fragments of HCV core that comprise, e.g., amino acids 10-45, 10-53, 67-88, and 120-130 of the polyprotein, epitope 5-1-1 (in the NS3 region of the viral genome) as well as defined epitopes derived from the E1, E2, c33c (NS3), c100 (NS4), NS3/4a and NS5 regions of the HCV polyprotein, as well as any of the other various epitopes identified from the HCV polyprotein. See, e.g., Chien et al., *Proc. Natl. Acad. Sci. USA* (1992) 89:10011-10015; Chien et al., *J. Gastroent. Hepatol.* (1993) 8:S33-39; Chien et al., International Publication No. WO 93/00365; Chien, D.Y., International Publication No. WO 94/01778; U.S. Patent Nos. 6,150,087 and 6,121,020, all of which are incorporated by reference herein.

The term "epitope" as used herein refers to a sequence of at least about 3 to 5, preferably about 5 to 10 or 15, and not more than about 1,000 amino acids (or any integer therebetween), which define a sequence that by itself or as part of a larger sequence, binds to an antibody generated in response to such sequence. There is no critical upper limit to the length of the fragment, which may comprise nearly the full-length of the protein sequence, or even a fusion protein comprising two or more epitopes from the HCV polyprotein. An epitope for use in the subject invention is not limited to

a polypeptide having the exact sequence of the portion of the parent protein from which it is derived. Indeed, viral genomes are in a state of constant flux and contain several variable domains which exhibit relatively high degrees of variability between isolates. Thus the term "epitope" encompasses sequences identical to the native sequence, as well as modifications to the native sequence, such as deletions, additions and substitutions (generally conservative in nature).

Regions of a given polypeptide that include an epitope can be identified using any number of epitope mapping techniques, well known in the art. See, e.g., *Epitope Mapping Protocols* in Methods in Molecular Biology, Vol. 66 (Glenn E. Morris, Ed., 1996) Humana Press, Totowa, New Jersey. For example, linear epitopes may be determined by e.g., concurrently synthesizing large numbers of peptides on solid supports, the peptides corresponding to portions of the protein molecule, and reacting the peptides with antibodies while the peptides are still attached to the supports. Such techniques are known in the art and described in, e.g., U.S. Patent No. 4,708,871; Geysen et al. (1984) *Proc. Natl. Acad. Sci. USA* 81:3998-4002; Geysen et al. (1985) *Proc. Natl. Acad. Sci. USA* 82:178-182; Geysen et al. (1986) *Molec. Immunol.* 23:709-715, all incorporated herein by reference in their entireties. Using such techniques, a number of epitopes of HCV have been identified. See, e.g., Chien et al., *Viral Hepatitis and Liver Disease* (1994) pp. 320-324, and further below. Similarly, conformational epitopes are readily identified by determining spatial conformation of amino acids such as by, e.g., x-ray crystallography and 2-dimensional nuclear magnetic resonance. See, e.g., *Epitope Mapping Protocols*, *supra*. Antigenic regions of proteins can also be identified using standard antigenicity and hydropathy plots, such as those calculated using, e.g., the Omiga version 1.0 software program available from the Oxford Molecular Group. This computer program employs the Hopp/Woods method, Hopp et al., *Proc. Natl. Acad. Sci USA* (1981) 78:3824-3828 for determining antigenicity profiles, and the Kyte-Doolittle technique, Kyte et al., *J. Mol. Biol.* (1982) 157:105-132 for hydropathy plots.

For a description of various HCV epitopes, see, e.g., Chien et al., *Proc. Natl. Acad. Sci. USA* (1992) 89:10011-10015; Chien et al., *J. Gastroent. Hepatol.* (1993) 8:S33-39; Chien et al., International Publication No. WO 93/00365; Chien, D.Y., International Publication No. WO 94/01778; and U.S. Patent Nos. 6,280,927 and 6,150,087, incorporated herein by reference in their entireties.

As used herein, the term "conformational epitope" refers to a portion of a full-length protein, or an analog or mutein thereof, having structural features native to the amino acid sequence encoding the epitope within the full-length natural protein. Native structural features include, but are not limited to, glycosylation and three dimensional structure. The length of the epitope-defining sequence can be subject to wide variations as these epitopes are believed to be formed by the three-dimensional shape of the antigen (e.g., folding). Thus, amino acids defining the epitope can be relatively few in number, but widely dispersed along the length of the molecule (or even on different molecules in the case of dimers, etc.), being brought into correct epitope conformation via folding. The portions of the antigen between the residues defining the epitope may not be critical to the conformational structure of the epitope. For example, deletion or substitution of these intervening sequences may not affect the conformational epitope provided sequences critical to epitope conformation are maintained (e.g., cysteines involved in disulfide bonding, glycosylation sites, etc.).

Conformational epitopes present in, e.g., the NS3/4a region are readily identified using methods discussed above. Moreover, the presence or absence of a conformational epitope in a given polypeptide can be readily determined through screening the antigen of interest with an antibody (polyclonal serum or monoclonal to the conformational epitope) and comparing its reactivity to that of a denatured version of the antigen which retains only linear epitopes (if any). In such screening using polyclonal antibodies, it may be advantageous to absorb the polyclonal serum first with the denatured antigen and see if it retains antibodies to the antigen of interest. Additionally, in the case of NS3/4a, a molecule which preserves the native conformation will also have protease and,

optionally, helicase enzymatic activities. Such activities can be detected using enzymatic assays, as described further below.

Preferably, a conformational epitope is produced recombinantly and is expressed in a cell from which it is extractable under conditions which preserve its desired structural features, e.g. without denaturation of the epitope. Such cells include bacteria, yeast, insect, and mammalian cells. Expression and isolation of recombinant conformational epitopes from the HCV polyprotein are described in e.g., International Publication Nos. WO 96/04301, WO 94/01778, WO 95/33053, WO 92/08734, which applications are incorporated by reference herein in their entireties. Alternatively, it is possible to express the antigens and further renature the protein after recovery. It is also understood that chemical synthesis may also provide conformational antigen mimitopes that cross-react with the "native" antigen's conformational epitope.

The term "multiple epitope fusion antigen" or "MEFA" as used herein intends a polypeptide in which multiple HCV antigens are part of a single, continuous chain of amino acids, which chain does not occur in nature. The HCV antigens may be connected directly to each other by peptide bonds or may be separated by intervening amino acid sequences. The fusion antigens may also contain sequences exogenous to the HCV polyprotein. Moreover, the HCV sequences present may be from multiple genotypes and/or isolates of HCV. Examples of particular MEFAs for use in the present immunoassays are detailed in, e.g., International Publication Nos. WO 01/96875, WO 01/09609, WO 97/44469 and U.S. Patent Nos. 6,514,731 and 6,428,792, incorporated herein by reference in their entireties.

An "antibody" intends a molecule that, through chemical or physical means, specifically binds to a polypeptide of interest. Thus, an HCV NS3/4a antibody is a molecule that specifically binds to an epitope of an HCV NS3/4a protein. The term "antibody" as used herein includes antibodies obtained from both polyclonal and monoclonal preparations, as well as, the following: hybrid (chimeric) antibody molecules (see, for example, Winter et al. (1991) *Nature* 349:293-299; and U.S. Patent

No. 4,816,567); F(ab')2 and F(ab) fragments; Fv molecules (non-covalent heterodimers, see, for example, Inbar et al. (1972) *Proc Natl Acad Sci USA* 69:2659-2662; and Ehrlich et al. (1980) *Biochem* 19:4091-4096); single-chain Fv molecules (sFv) (see, for example, Huston et al. (1988) *Proc Natl Acad Sci USA* 85:5879-5883); dimeric and trimeric antibody fragment constructs; minibodies (see, e.g., Pack et al. (1992) *Biochem* 31:1579-1584; Cumber et al. (1992) *J Immunology* 149B:120-126); humanized antibody molecules (see, for example, Riechmann et al. (1988) *Nature* 332:323-327; Verhoeyan et al. (1988) *Science* 239:1534-1536; and U.K. Patent Publication No. GB 2,276,169, published 21 September 1994); and, any functional fragments obtained from such molecules, wherein such fragments retain immunological binding properties of the parent antibody molecule.

A "recombinant" protein is a protein which retains the desired activity and which has been prepared by recombinant DNA techniques as described herein. In general, the gene of interest is cloned and then expressed in transformed organisms, as described further below. The host organism expresses the foreign gene to produce the protein under expression conditions.

By "isolated" is meant, when referring to a polypeptide, that the indicated molecule is separate and discrete from the whole organism with which the molecule is found in nature or is present in the substantial absence of other biological macro-molecules of the same type. The term "isolated" with respect to a polynucleotide is a nucleic acid molecule devoid, in whole or part, of sequences normally associated with it in nature; or a sequence, as it exists in nature, but having heterologous sequences in association therewith; or a molecule disassociated from the chromosome.

By "equivalent antigenic determinant" is meant an antigenic determinant from different sub-species or strains of HCV, such as from strains 1, 2, 3, etc. of HCV. More specifically, epitopes are known, such as 5-1-1, and such epitopes vary between the strains 1, 2, and 3. Thus, the epitope 5-1-1 from the three different strains are equivalent antigenic determinants and thus are "copies" even though their sequences are not

identical. In general the amino acid sequences of equivalent antigenic determinants will have a high degree of sequence homology, e.g., amino acid sequence homology of more than 30%, preferably more than 40%, when the two sequences are aligned.

"Homology" refers to the percent similarity between two polynucleotide or two polypeptide moieties. Two DNA, or two polypeptide sequences are "substantially homologous" to each other when the sequences exhibit at least about 50% , preferably at least about 75%, more preferably at least about 80%-85%, preferably at least about 90%, and most preferably at least about 95%-98% sequence similarity over a defined length of the molecules. As used herein, substantially homologous also refers to sequences showing complete identity to the specified DNA or polypeptide sequence.

In general, "identity" refers to an exact nucleotide-to-nucleotide or amino acid-to-amino acid correspondence of two polynucleotides or polypeptide sequences, respectively. Percent identity can be determined by a direct comparison of the sequence information between two molecules by aligning the sequences, counting the exact number of matches between the two aligned sequences, dividing by the length of the shorter sequence, and multiplying the result by 100.

Readily available computer programs can be used to aid in the analysis of similarity and identity, such as ALIGN, Dayhoff, M.O. in *Atlas of Protein Sequence and Structure* M.O. Dayhoff ed., 5 Suppl. 3:353-358, National biomedical Research Foundation, Washington, DC, which adapts the local homology algorithm of Smith and Waterman *Advances in Appl. Math.* 2:482-489, 1981 for peptide analysis. Programs for determining nucleotide sequence similarity and identity are available in the Wisconsin Sequence Analysis Package, Version 8 (available from Genetics Computer Group, Madison, WI) for example, the BESTFIT, FASTA and GAP programs, which also rely on the Smith and Waterman algorithm. These programs are readily utilized with the default parameters recommended by the manufacturer and described in the Wisconsin Sequence Analysis Package referred to above. For example, percent similarity of a particular nucleotide sequence to a reference sequence can be determined using the

homology algorithm of Smith and Waterman with a default scoring table and a gap penalty of six nucleotide positions.

Another method of establishing percent similarity in the context of the present invention is to use the MPSRCH package of programs copyrighted by the University of Edinburgh, developed by John F. Collins and Shane S. Sturrok, and distributed by IntelliGenetics, Inc. (Mountain View, CA). From this suite of packages the Smith-Waterman algorithm can be employed where default parameters are used for the scoring table (for example, gap open penalty of 12, gap extension penalty of one, and a gap of six). From the data generated the "Match" value reflects "sequence similarity." Other suitable programs for calculating the percent identity or similarity between sequences are generally known in the art, for example, another alignment program is BLAST, used with default parameters. For example, BLASTN and BLASTP can be used using the following default parameters: genetic code = standard; filter = none; strand = both; cutoff = 60; expect = 10; Matrix = BLOSUM62; Descriptions = 50 sequences; sort by = HIGH SCORE; Databases = non-redundant, GenBank + EMBL + DDBJ + PDB + GenBank CDS translations + Swiss protein + Spupdate + PIR. Details of these programs can be found at the following internet address: http://www.ncbi.nlm.gov/cgi-bin/BLAST.

Alternatively, homology can be determined by hybridization of polynucleotides under conditions which form stable duplexes between homologous regions, followed by digestion with single-stranded-specific nuclease(s), and size determination of the digested fragments. DNA sequences that are substantially homologous can be identified in a Southern hybridization experiment under, for example, stringent conditions, as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Sambrook et al., *supra*; *DNA Cloning*, *supra*; *Nucleic Acid Hybridization*, *supra*.

"Common solid support" intends a single solid matrix to which the HCV polypeptides used in the subject immunoassays are bound covalently or by noncovalent means such as hydrophobic adsorption.

"Immunologically reactive" means that the antigen in question will react specifically with anti-HCV antibodies present in a biological sample from an HCV-infected individual.

"Immune complex" intends the combination formed when an antibody binds to an epitope on an antigen.

As used herein, a "biological sample" refers to a sample of tissue or fluid isolated from a subject, including but not limited to, for example, blood, plasma, serum, fecal matter, urine, bone marrow, bile, spinal fluid, lymph fluid, samples of the skin, external secretions of the skin, respiratory, intestinal, and genitourinary tracts, tears, saliva, milk, blood cells, organs, biopsies and also samples of *in vitro* cell culture constituents including but not limited to conditioned media resulting from the growth of cells and tissues in culture medium, e.g., recombinant cells, and cell components.

As used herein, the terms "label" and "detectable label" refer to a molecule capable of detection, including, but not limited to, radioactive isotopes, fluorescers, chemiluminescers, chromophores, enzymes, enzyme substrates, enzyme cofactors, enzyme inhibitors, chromophores, dyes, metal ions, metal sols, ligands (e.g., biotin, strepavidin or haptens) and the like. The term "fluorescer" refers to a substance or a portion thereof which is capable of exhibiting fluorescence in the detectable range. Particular examples of labels which may be used under the invention include, but are not limited to, horse radish peroxidase (HRP), fluorescein, FITC, rhodamine, dansyl, umbelliferone, dimethyl acridinium ester (DMAE), Texas red, luminol, NADPH and α-β-galactosidase.

II. Modes of Carrying out the Invention

Before describing the present invention in detail, it is to be understood that this invention is not limited to particular formulations or process parameters as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments of the invention only, and is not intended to be limiting.

Although a number of compositions and methods similar or equivalent to those described herein can be used in the practice of the present invention, the preferred materials and methods are described herein.

As noted above, the present invention is based on the discovery of novel antigen/antibody/antigen sandwich diagnostic methods for accurately detecting HCV infection. The methods can be practiced quickly and efficiently and eliminate background effects that can occur from the use of large sample volumes. The methods preferably utilize highly immunogenic HCV antigens which are present during the early stages of HCV seroconversion, thereby increasing detection accuracy and reducing the incidence of false results.

In particular, the immunoassays described herein utilize two basic HCV antigens, one present in the solid phase and one present in the solution phase. Both antigens are capable of binding HCV antibodies present in biological samples from infected individuals. One antigen used in the subject assays is an isolated antigen from a region of the HCV polyprotein. The second antigen used is a multiple epitope fusion antigen ("MEFA") comprising various HCV polypeptides, either from the same or different HCV genotypes and isolates. The MEFA includes at least one or more epitopes derived from the same region of the HCV polyprotein as the isolated HCV antigen.

In particularly preferred embodiments, one of the antigens used in the subject assays is a highly immunogenic conformational epitope derived from the NS3/4a region of the HCV polyprotein. In these embodiments, the second antigen used is a MEFA which includes one or more epitopes from the NS3/4a region (either linear or

conformational), as described further below. The MEFA may therefore include multiple immunodominant epitopes derived from the NS3/4a region from one or more HCV isolates. If multiple NS3/4a epitopes are used in the multiple epitope fusion, they may be the same or different epitopes. Alternatively, the fusion antigen may include one or more epitopes derived from the NS3/4a region, as well as major linear epitopes from other HCV regions such as, without limitation, HCV core, E1, E2, P7, NS4b, NS5a and NS5b sequences.

The methods can be conveniently practiced in a single assay, using any of the several assay formats described below, such as but not limited to, assay formats which utilize a solid support to which either the isolated HCV antigen, such as the NS3/4a conformational epitope, or the MEFA, is bound. Thus, the MEFA can be provided in either the solution or the solid phase. If provided in solution, the isolated HCV antigen is present on the solid phase.

For example, in one representative method of the invention, the assay is conducted on a solid support to which has been bound one or more polypeptides including one or more conformational epitopes derived from the NS3/4a region of the HCV polyprotein. In this embodiment, the MEFA is provided in the solution phase. In an alternative embodiment, the assay is conducted on a solid support to which one or more MEFAs has been bound. In this embodiment, the polypeptide including the conformational NS3/4a epitope is provided in the solution phase. Thus, if the conformational NS3/4a epitope is present on the solid support, the MEFA is present in the solution phase, and vice versa.

In order to further an understanding of the invention, a more detailed discussion is provided below regarding the various HCV polypeptide antigens and MEFAs for use in the subject methods, as well as production of the proteins and methods of using the proteins.

HCV Antigens and MEFAs

The genomes of HCV strains contain a single open reading frame of approximately 9,000 to 12,000 nucleotides, which is transcribed into a polyprotein. As shown in Figure 1 and Table 1, an HCV polyprotein, upon cleavage, produces at least ten distinct products, in the order of NH_2- Core-E1-E2-p7-NS2-NS3-NS4a-NS4b-NS5a-NS5b-COOH. The core polypeptide occurs at positions 1-191, numbered relative to HCV-1 (see, Choo et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:2451-2455, for the HCV-1 genome). This polypeptide is further processed to produce an HCV polypeptide with approximately amino acids 1-173. The envelope polypeptides, E1 and E2, occur at about positions 192-383 and 384-746, respectively. The P7 domain is found at about positions 747-809. NS2 is an integral membrane protein with proteolytic activity and is found at about positions 810-1026 of the polyprotein. NS2, in combination with NS3, (found at about positions 1027-1657), cleaves the NS2-NS3 sissle bond which in turn generates the NS3 N-terminus and releases a large polyprotein that includes both serine protease and RNA helicase activities. The NS3 protease, found at about positions 1027-1207, serves to process the remaining polyprotein. The helicase activity is found at about positions 1193-1657. NS3 liberates an NS3 cofactor (NS4a, found about positions 1658-1711), two proteins (NS4b found at about positions 1712-1972, and NS5a found at about positions 1973-2420), and an RNA-dependent RNA polymerase (NS5b found at about positions 2421-3011). Completion of polyprotein maturation is initiated by autocatalytic cleavage at the NS3-Ns4a junction, catalyzed by the NS3 serine protease.

Table 1	
Domain	Approximate Boundaries*
C (core)	1-191
E1	192-383
E2	384-746
P7	747-809
NS2	810-1026
NS3	1027-1657
NS4a	1658-1711
NS4b	1712-1972
NS5a	1973-2420
NS5b	2421-3011

*Numbered relative to HCV-1. See, Choo et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:2451-2455.

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One component of the subject methods is an isolated antigen from any of the various regions of the HCV polyprotein as described above. Nucleic acid and amino

acid sequences of a number of HCV strains and isolates, including nucleic acid and amino acid sequences of the various regions described above have been determined. For example, isolate HCV J1.1 is described in Kubo et al. (1989) *Japan. Nucl. Acids Res.* 17:10367-10372; Takeuchi et al. (1990) *Gene* 91:287-291; Takeuchi et al. (1990) *J. Gen. Virol.* 71:3027-3033; and Takeuchi et al. (1990) *Nucl. Acids Res.* 18:4626. The complete coding sequences of two independent isolates, HCV-J and BK, are described by Kato et al., (1990) *Proc. Natl. Acad. Sci. USA* 87:9524-9528 and Takamizawa et al., (1991) *J. Virol.* 65:1105-1113 respectively.

Publications that describe HCV-1 isolates include Choo *et al.* (1990) Brit. Med. Bull. 46:423-441; Choo et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:2451-2455 and Han et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:1711-1715. HCV isolates HC-J1 and HC-J4 are described in Okamoto et al. (1991) *Japan J. Exp. Med.* 60:167-177. HCV isolates HCT 18, HCT 23, Th, HCT 27, EC1 and EC10 are described in Weiner et al. (1991) *Virol.* 180:842-848. HCV isolates Pt-1, HCV-K1 and HCV-K2 are described in Enomoto et al. (1990) *Biochem. Biophys. Res. Commun.* 170:1021-1025. HCV isolates A, C, D & E are described in Tsukiyama-Kohara et al. (1991) *Virus Genes* 5:243-254.

Thus, for example, the isolated HCV antigen can be derived from the core region of any of these HCV isolates. This region occurs at amino acid positions 1-191 of the HCV polyprotein, numbered relative to HCV-1. Either the full-length protein, fragments thereof, such as amino acids 1-150, e.g., amino acids 1-130, 1-120, for example, amino acids 1-121, 1-122, 1-123, etc., or smaller fragments containing epitopes of the full-length protein may be used in the subject methods, such as those epitopes found between amino acids 10-53, amino acids 10-45, amino acids 67-88, amino acids 120-130, or any of the core epitopes identified in, e.g., Houghton et al., U.S. Patent No. 5,350,671; Chien et al., *Proc. Natl. Acad. Sci. USA* (1992) 89:10011-10015; Chien et al., *J. Gastroent. Hepatol.* (1993) 8:S33-39; Chien et al., International Publication No. WO 93/00365; Chien, D.Y., International Publication No. WO 94/01778; and U.S. Patent Nos. 6,280,927 and 6,150,087, the disclosures of which are incorporated herein by reference

in their entireties. Moreover, a protein resulting from a frameshift in the core region of the polyprotein, such as described in International Publication No. WO 99/63941, may be used.

Similarly, polypeptides from the HCV E1 and/or E2 regions can be used in the methods of the present invention as the isolated HCV antigen. E2 exists as multiple species (Spaete et al., *Virol.* (1992) 188:819-830; Selby et al., *J. Virol.* (1996) 70:5177-5182; Grakoui et al., *J. Virol.* (1993) 67:1385-1395; Tomei et al., *J. Virol.* (1993) 67:4017-4026) and clipping and proteolysis may occur at the NB and C-termini of the E2 polypeptide. Thus, an E2 polypeptide for use herein may comprise amino acids 405-661, e.g., 400, 401, 402... to 661, such as 383 or 384-661, 383 or 384-715, 383 or 384-746, 383 or 384-749 or 383 or 384-809, or 383 or 384 to any C-terminus between 661-809, of an HCV polyprotein, numbered relative to the full-length HCV-1 polyprotein. Similarly, E1 polypeptides for use herein can comprise amino acids 192-326, 192-330, 192-333, 192-360, 192-363, 192-383, or 192 to any C-terminus between 326-383, of an HCV polyprotein.

Immunogenic fragments of E1 and/or E2 which comprise epitopes may be used in the subject methods. For example, fragments of E1 polypeptides can comprise from about 5 to nearly the full-length of the molecule, such as 6, 10, 25, 50, 75, 100, 125, 150, 175, 185 or more amino acids of an E1 polypeptide, or any integer between the stated numbers. Similarly, fragments of E2 polypeptides can comprise 6, 10, 25, 50, 75, 100, 150, 200, 250, 300, or 350 amino acids of an E2 polypeptide, or any integer between the stated numbers.

For example, epitopes derived from, e.g., the hypervariable region of E2, such as a region spanning amino acids 384-410 or 390-410, can be included in the fusions. A particularly effective E2 epitope to incorporate into an E2 polypeptide sequence is one which includes a consensus sequence derived from this region, such as the consensus sequence Gly-Ser-Ala-Ala-Arg-Thr-Thr-Ser-Gly-Phe-Val-Ser-Leu-Phe-Ala-Pro-Gly-Ala-Lys-Gln-Asn (SEQ ID NO:7), which represents a consensus sequence for amino

acids 390-410 of the HCV type 1 genome. Additional epitopes of E1 and E2 are known and described in, e.g., Chien et al., International Publication No. WO 93/00365.

Moreover, the E1 and/or E2 polypeptides may lack all or a portion of the membrane spanning domain. With E1, generally polypeptides terminating with about amino acid position 370 and higher (based on the numbering of HCV-1 E1) will be retained by the ER and hence not secreted into growth media. With E2, polypeptides terminating with about amino acid position 731 and higher (also based on the numbering of the HCV-1 E2 sequence) will be retained by the ER and not secreted. (See, e.g., International Publication No. WO 96/04301, published February 15, 1996). It should be noted that these amino acid positions are not absolute and may vary to some degree. Thus, the present invention contemplates the use of E1 and/or E2 polypeptides which retain the transmembrane binding domain, as well as polypeptides which lack all or a portion of the transmembrane binding domain, including E1 polypeptides terminating at about amino acids 369 and lower, and E2 polypeptides, terminating at about amino acids 730 and lower. Furthermore, the C-terminal truncation can extend beyond the transmembrane spanning domain towards the N-terminus. Thus, for example, E1 truncations occurring at positions lower than, e.g., 360 and E2 truncations occurring at positions lower than, e.g., 715, are also encompassed by the present invention. All that is necessary is that the truncated E1 and E2 polypeptides remain functional for their intended purpose. However, particularly preferred truncated E1 constructs are those that do not extend beyond about amino acid 300. Most preferred are those terminating at position 360. Preferred truncated E2 constructs are those with C-terminal truncations that do not extend beyond about amino acid position 715. Particularly preferred E2 truncations are those molecules truncated after any of amino acids 715-730, such as 725.

Additionally, epitopes from the NS3, NS4, NS5a, or NS5b regions can be used as the isolated HCV antigen. Particularly preferred, is the use of an epitope from the NS3/4a region. The NS3/4a region of the HCV polyprotein has been described and the amino acid sequence and overall structure of the protein are disclosed in, e.g., Yao et al.,

Structure (November 1999) 7:1353-1363; Sali et al., *Biochem.* (1998) 37:3392-3401; and Bartenschlager, R., *J. Viral Hepat.* (1999) 6:165-181. See, also, Dasmahapatra et al., U.S. Patent No. 5,843,752, incorporated herein by reference in its entirety. The subject immunoassays can utilize at least one conformational epitope derived from the NS3/4a region that exists in the conformation as found in the naturally occurring HCV particle or its infective product, as evidenced by the preservation of protease and, optionally, helicase enzymatic activities normally displayed by the NS3/4a gene product and/or immunoreactivity of the antigen with antibodies in a biological sample from an HCV-infected subject, and a loss of the epitope's immunoreactivity upon denaturation of the antigen. For example, the conformational epitope can be disrupted by heating, changing the pH to extremely acid or basic, or by adding known organic denaturants, such as dithiothreitol (DTT) or an appropriate detergent. See, e.g., *Protein Purification Methods, a practical approach* (E.L.V. Harris and S. Angal eds., IRL Press) and the denatured product compared to the product which is not treated as above.

Protease and helicase activity may be determined using standard enzyme assays well known in the art. For example, protease activity may be determined using assays well known in the art. See, e.g., Takeshita et al., *Anal. Biochem.* (1997) 247:242-246; Kakiuchi et al., *J. Biochem.* (1997) 122:749-755; Sali et al., *Biochemistry* (1998) 37:3392-3401; Cho et al., *J. Virol. Meth.* (1998) 72:109-115; Cerretani et al., *Anal. Biochem.* (1999) 266:192-197; Zhang et al., *Anal. Biochem.* (1999) 270:268-275; Kakiuchi et al., *J. Virol. Meth.* (1999) 80:77-84; Fowler et al., *J. Biomol. Screen.* (2000) 5:153-158; and Kim et al., *Anal. Biochem.* (2000) 284:42-48. A particularly convenient assay for testing protease activity is set forth in the examples below.

Similarly, helicase activity assays are well known in the art and helicase activity of an NS3/4a epitope may be determined using, for example, an ELISA assay, as described in, e.g., Hsu et al., *Biochem. Biophys. Res. Commun.* (1998) 253:594-599; a scintillation proximity assay system, as described in Kyono et al., *Anal. Biochem.* (1998) 257:120-126; high throughput screening assays as described in, e.g., Hicham et al.,

Antiviral Res. (2000) 46:181-193 and Kwong et al., *Methods Mol. Med.* (2000) 24:97-116; as well as by other assay methods known in the art. See, e.g., Khu et al., *J. Virol.* (2001) 75:205-214; Utama et al., *Virology* (2000) 273:316-324; Paolini et al., *J. Gen. Virol.* (2000) 81:1335-1345; Preugschat et al., *Biochemistry* (2000) 39:5174-5183; Preugschat et al., *Methods Mol. Med.* (1998) 19:353-364; and Hesson et al., *Biochemistry* (2000) 39:2619-2625.

If a conformational NS3/4a epitope is used, the length of the antigen is sufficient to maintain an immunoreactive conformational epitope. Often, the polypeptide containing the antigen used will be almost full-length, however, the polypeptide may also be truncated to, for example, increase solubility or to improve secretion. Generally, the conformational epitope found in NS3/4a is expressed as a recombinant polypeptide in a cell and this polypeptide provides the epitope in a desired form, as described in detail below.

A representative amino acid sequence for an NS3/4a polypeptide is shown in Figures 3A through 3D (SEQ ID NOS:1 and 2). The amino acid sequence shown at positions 2-686 of Figures 3A through 3D corresponds to amino acid positions 1027-1711 of HCV-1. An initiator codon (ATG) coding for Met, is shown as position 1. Additionally, the Thr normally occurring at position 1428 of HCV-1 (amino acid position 403 of Figure 3) is mutated to Pro, and the Ser normally occurring at position 1429 of HCV-1 (amino acid position 404 of Figure 3) is mutated to Ile. However, either the native sequence, with or without an N-terminal Met, the depicted analog, with or without the N-terminal Met, or other analogs and fragments can be used in the subject assays, so long as the epitope is produced using a method that retains or reinstates its native conformation such that protease activity, and optionally, helicase activity is retained. Dasmahapatra et al., U.S. Patent No. 5,843,752 and Zhang et al., U.S. Patent No. 5,990,276, both describe analogs of NS3/4a.

The NS3 protease of NS3/4a is found at about positions 1027-1207, numbered relative to HCV-1, positions 2-182 of Figure 3. The structure of the NS3 protease and

active site are known. See, e.g., De Francesco et al., *Antivir. Ther.* (1998) 3:99-109; Koch et al., *Biochemistry* (2001) 40:631-640. Changes to the native sequence that will normally be tolerated will be those outside of the active site of the molecule. Particularly, it is desirable to maintain amino acids 1- or 2-155 of Figure 3, with little or only conservative substitutions. Amino acids occurring beyond position 155 will tolerate greater changes. Additionally, if fragments of the NS3/4a sequence are used, these fragments will generally include at least amino acids 1- or 2-155, preferably amino acids 1- or 2-175, and most preferably amino acids 1- or 2-182, with or without the N-terminal Met. The helicase domain is found at about positions 1193-1657 of HCV-1 (positions 207-632 of Figure 3). Thus, if helicase activity is desired, this portion of the molecule will be maintained with little or only conservative changes. One of skill in the art can readily determine other regions that will tolerate change based on the known structure of NS3/4a.

A number of antigens including epitopes derived from NS3/4a are known, including, but not limited to antigens derived from the c33c, c200, c100 and 5-1-1 regions, as well as fusion proteins comprising an NS3 epitope, such as c25.

For a description of these and various other HCV epitopes from other HCV regions, see, e.g., Houghton et al, U.S. Patent No. 5,350,671; Chien et al., *Proc. Natl. Acad. Sci. USA* (1992) 89:10011-10015; Chien et al., *J. Gastroent. Hepatol.* (1993) 8:S33-39; Chien et al., International Publication No. WO 93/00365; Chien, D.Y., International Publication No. WO 94/01778; and U.S. Patent Nos. 6,280,927 and 6,150,087, incorporated herein by reference in their entireties.

The immunoassays described herein also utilize multiple epitope fusion antigens (termed "MEFAs"), as described in International Publication Nos. WO 01/96875, WO 01/09609, WO 97/44469 and U.S. Patent Nos. 6,514,731 and 6,428,792, incorporated herein by reference in their entireties. The MEFAs for use in the subject assays include multiple epitopes derived from any of the various viral regions shown in Figure 1 and Table 1, and as described above.

The multiple HCV antigens are part of a single, continuous chain of amino acids, which chain does not occur in nature. Thus, the linear order of the epitopes is different than their linear order in the genome in which they occur. The linear order of the sequences of the MEFAs for use herein is preferably arranged for optimum antigenicity. Preferably, the epitopes are from more than one HCV strain, thus providing the added ability to detect multiple strains of HCV in a single assay. Thus, the MEFAs for use herein may comprise various immunogenic regions derived from the polyprotein described above.

As explained above, in a particularly preferred embodiment, an NS3/4a epitope is used as the isolated HCV antigen. In this embodiment, the MEFA includes at least one or more epitopes derived from the NS3/4a region (either linear or conformational). The MEFA may therefore include multiple immunodominant epitopes derived from the NS3/4a region from one or more HCV isolates. If multiple NS3/4a epitopes are used in the multiple epitope fusion, they may be the same or different epitopes. Alternatively, the fusion antigen may include one or more epitopes derived from the NS3/4a region, as well as major linear epitopes from other HCV regions such as, without limitation, HCV core, E1, E2, P7, NS4b, NS5a and NS5b sequences.

Polypeptides comprising epitopes derived from the NS3/4a region include, without limitation, polypeptides comprising all or a portion of the NS3, NS4a and NS3/4a regions. A number of epitopes from these regions are known, including, but not limited to antigens derived from the c33c, c200 and c100 regions, as well as fusion proteins comprising an NS3 epitope, such as c25. These and other NS3 epitopes are useful in the present assays and are known in the art and described in, e.g., Houghton et al, U.S. Patent No. 5,350,671; Chien et al., *Proc. Natl. Acad. Sci. USA* (1992) 89:10011-10015; Chien et al., *J. Gastroent. Hepatol.* (1993) 8:S33-39; Chien et al., International Publication No. WO 93/00365; Chien, D.Y., International Publication No. WO 94/01778; and U.S. Patent Nos. 6,346,375 and 6,150,087, the disclosures of which are incorporated herein by reference in their entireties.

Moreover, the antigenic determinant known as 5-1-1 is partially within the NS4a region (see, Figure 1) and is particularly useful in the MEFAs for use in the subject assays. This antigenic determinant appears in three different forms on three different viral strains of HCV. Accordingly, in a preferred embodiment of the invention all three forms of 5-1-1 appear on the multiple epitope fusion antigen used in the subject immunoassays.

Additional HCV epitopes for use in the MEFAs include any of the various epitopes described above, such as epitopes derived from the hypervariable region of E2, such as a region spanning amino acids 384-410 or 390-410, or the consensus sequence from this region. as described above (Gly-Ser-Ala-Ala-Arg-Thr-Thr-Ser-Gly-Phe-Val-Ser-Leu-Phe-Ala-Pro-Gly-Ala-Lys-Gln-Asn) (SEQ ID NO: 7), which represents a consensus sequence for amino acids 390-410 of the HCV type 1 genome. A representative E2 epitope present in a MEFA of the invention can comprise a hybrid epitope spanning amino acids 390-444. Such a hybrid E2 epitope can include a consensus sequence representing amino acids 390-410 fused to the native amino acid sequence for amino acids 411-444 of HCV E2.

As explained above, the antigens may be derived from various HCV strains. Multiple viral strains of HCV are known, and epitopes derived from any of these strains can be used in a fusion protein. It is well known that any given species of organism varies from one individual organism to another and further that a given organism such as a virus can have a number of different strains. For example, as explained above, HCV includes at least 6 genotypes. Each of these genotypes includes equivalent antigenic determinants. More specifically, each strain includes a number of antigenic determinants that are present on all strains of the virus but are slightly different from one viral strain to another. Similarly, equivalent antigenic determinants from the core region of different HCV strains may also be present. In general, equivalent antigenic determinants have a high degree of homology in terms of amino acid sequence which degree of homology is generally 30% or more, preferably 40% or more, when aligned.

The multiple copy epitope of the present invention can also include multiple copies which are exact copies of the same epitope.

Representative MEFAs for use with the present assays are shown in Figures 5, 7 and 9A-9C, and are described in International publication nos. WO 01/96875, WO 01/09609, WO 97/44469 and U.S. Patent Nos. 6,514,731 and 6,428,792, incorporated herein by reference in their entireties. Representative MEFAs for use herein include those termed MEFA 3, MEFA 5, MEFA 6, MEFA 7.1, MEFA 12, MEFA 13 and MEFA 13.1. It is to be understood that these MEFAs are merely representative and other epitopes derived from the HCV genome will also find use with the present assays and may be incorporated into these or other MEFAs.

The DNA sequence and corresponding amino acid sequence of MEFA 12 is shown in Figures 6A through 6F (SEQ ID NOS:3 and 4). The general structural formula for MEFA 12 is shown in Figure 5 and is as follows: hSOD-E1(type 1)-E2 HVR consensus(type 1a)-E2 HVR consensus(types 1 and 2)-c33c short(type 1)-5-1-1(type 1)-5-1-1(type 3)-5-1-1(type 2)-c100(type 1)-NS5(type 1)-NS5(type 1)-core(types 1+2)-core(types 1+2). This multiple copy epitope includes the following amino acid sequence, numbered relative to HCV-1 (the numbering of the amino acids set forth below follows the numbering designation provided in Choo, et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:2451-2455, in which amino acid #1 is the first methionine encoded by the coding sequence of the core region): amino acids 1-69 of superoxide dismutase (truncated SOD, used to enhance recombinant expression of the protein); amino acids 303 to 320 of the polyprotein from the E1 region; amino acids 390 to 410 of the polyprotein, representing a consensus sequence for the hypervariable region of HCV-1a E2; amino acids 384 to 414 of the polyprotein from region E2, representing a consensus sequence for the E2 hypervariable regions of HCV-1 and HCV-2; amino acids 1211-1457 of the HCV-1 polyprotein which define the helicase; three copies of an epitope from 5-1-1, amino acids 1689-1735, one from HCV-1, one from HCV-3 and one from HCV-2, which copies are equivalent antigenic determinants from the three different viral

strains of HCV; HCV polypeptide c100 of HCV-1, amino acids 1901-1936 of the polyprotein; two exact copies of an epitope from the NS5 region of HCV-1, each with amino acids 2278 to 2313 of the HCV polyprotein; and two copies of three epitopes from the core region, two from HCV-1 and one from HCV-2, which copies are equivalent antigenic determinants represented by amino acids 9 to 53 and 64-88 of HCV-1 and 67-84 of HCV-2.

Table 2 shows the amino acid positions of the various epitopes in MEFA 12 with reference to Figures 6A through 6F herein (SEQ ID NOS:3 and 4). The numbering in the tables is relative to HCV-1. See, Choo et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:2451-2455. MEFAs 13 and 13.1 also share the general formula specified above for MEFA 12, with modifications as indicated in Tables 3 and 4, respectively.

Table 2. MEFA 12				
mefa aa#	5' end site	epitope	hcv aa#	strain
1-69	*Nco*1	truncated hSOD		
72-89	*Mlu*I	E1	303-320	1
92-112	*Hind*111	E2 HVR1a consensus	390-410	1
113-143		E2 HVR1+2 consensus	384-414	1, 2
146-392	*Spe*I	C33C short	1211-1457	1
395-441	*Sph*I	5-1-1	1689-1735	1
444-490	*Nru*I	5-1-1	1689-1735	3
493-539	*Cla*I	5-1-1	1689-1735	2
542-577	*Ava*I	C100	1901-1936	1
580-615	*Xba*I	NS5	2278-2313	1
618-653	*Bgl*II	NS5	2278-2313	1
654-741	*Nco*I	core epitopes	9-53, R47L 64-88 67-84	1 1 2
742-829	*Bal*I	core epitopes	9-53, R47L 64-88 67-84	1 1 2

Table 3. MEFA 13				
mefa aa#	5' end site	epitope	hcv aa#	strain
1-156	*Nco*1	mutated hSOD (aa 70-72, ALA)		
161-178	*Mlu*I	E1	303-320	1
181-201	*Hind*111	E2 HVR1a consensus	390-410	1
202-232		E2 HVR1+2 consensus	384-414	1, 2
235-451		C33C short	1211-1457	1
454-500	*Hind*III	5-1-1 PImut*	1689-1735	1
503-549	*Nru*I	5-1-1 PImut*	1689-1735	3
552-598	*Cla*I	5-1-1 PImut*	1689-1735	2
601-636	*Ava*I	C100	1901-1936	1
639-674	*Xba*I	NS5	2278-2313	1
677-712	*Bgl*II	NS5	2278-2313	1
713-800		core epitopes	9-53, R47L 64-88 67-84	1 1 2
801-888		core epitopes	9-53, R47L 64-88 67-84	1 1 2

*The 5-1-1 epitopes are modified by eliminating possible cleavage sites (CS or CA) targeted by the NS3/4a recombinant protein. Instead of CS or CA, the sequence has been changed to PI.

Table 4. MEFA 13.1				
mefa aa#	5' end site	epitope	hcv aa#	strain
1-86	*Nco*I	mutated hSOD (aa 70-72, ALA)		
89-106	*Mlu*I	E1	303-320	1
109-129	*Hind*III	E2 HVR1a consensus	390-410	1
130-160		E2 HVR1+2 consensus	384-414	1, 2
163-379		C33C short	1211-1457	1
382-428	*Hind*III	5-1-1 PImut*	1689-1735	1
431-477	*Nru*I	5-1-1 PImut*	1689-1735	3
480-526	*Cla*I	5-1-1 PImut*	1689-1735	2
529-564	*Ava*I	C100	1901-1936	1
567-602	*Xba*I	NS5	2278-2313	1
605-640	*Bgl*II	NS5	2278-2313	1
641-728		core epitopes	9-53, R47L 64-88 67-84	1 1 2
729-816		core epitopes	9-53, R47L 64-88 67-84	1 1 2

*The 5-1-1 epitopes are modified by eliminating possible cleavage sites (CS or CA) targeted by the NS3/4a recombinant protein. Instead of CS or CA, the sequence has been changed to PI.

The DNA sequence and corresponding amino acid sequence of another representative multiple epitope fusion antigen, MEFA 7.1, is shown in Figures 8A

through 8F (SEQ ID NOS:5 and 6). The general structural formula for MEFA 7.1 is shown in Figure 7 and is as follows: hSOD-E1(type 1)-E2 HVR consensus(type 1a)-E2 HVR consensus(types 1 and 2)-helicase(type 1)-5-1-1(type 1)-5-1-1(type 3)-5-1-1(type 2)-c100(type 1)-NS5(type 1)-NS5(type 1)-core(types 1+2)-core(types 1+2). This multiple copy epitope includes the following amino acid sequence, numbered relative to HCV-1 (the numbering of the amino acids set forth below follows the numbering designation provided in Choo, et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:2451-2455, in which amino acid #1 is the first methionine encoded by the coding sequence of the core region): amino acids 1-156 of superoxide dismutase (SOD, used to enhance recombinant expression of the protein); amino acids 303 to 320 of the polyprotein from the E1 region; amino acids 390 to 410 of the polyprotein, representing a consensus sequence for the hypervariable region of HCV-1a E2; amino acids 384 to 414 of the polyprotein from region E2, representing a consensus sequence for the E2 hypervariable regions of HCV-1 and HCV-2; amino acids 1193-1658 of the HCV-1 polyprotein which define the helicase; three copies of an epitope from 5-1-1, amino acids 1689-1735, one from HCV-1, one from HCV-3 and one from HCV-2, which copies are equivalent antigenic determinants from the three different viral strains of HCV; HCV polypeptide C100 of HCV-1, amino acids 1901-1936 of the polyprotein; two exact copies of an epitope from the NS5 region of HCV-1, each with amino acids 2278 to 2313 of the HCV polyprotein; and two copies of an epitope from the core region, one from HCV-1 and one from HCV-2, which copies are equivalent antigenic determinants represented by amino acids 9 to 32, 39-42 and 64-88 of HCV-1 and 67-84 of HCV-2.

Table 5 shows the amino acid positions of the various epitopes with reference to Figures 8A through 8F herein (SEQ ID NOS:5 and 6).

Table 5. MEFA 7.1				
mefa aa#	5' end site	epitope	hcv aa#	strain
1-156	*Nco*1	hSOD		
159-176	*Eco*R1	E1	303-320	1
179-199	*Hind*111	E2 HVR1a consensus	390-410	1
200-230		E2 HVR1+2 consensus	384-414	1+2
231-696	*Sal*1	Helicase	1193-1658	1
699-745	*Sph*1	5-1-1	1689-1735	1
748-794	*Nru*1	5-1-1	1689-1735	3
797-843	*Cla*1	5-1-1	1689-1735	2
846-881	*Ava*1	C100	1901-1936	1
884-919	*Xba*1	NS5	2278-2313	1
922-957	*Bgl*11	NS5	2278-2313	1
958-1028	*Nco*1	core epitopes	9-32, 39-42 64-88 67-84	1 1 2
1029-1099	*Bal*1	core epitopes	9-32, 39-42, 64-88 67-84	1 1 2

In one assay format, the sample is combined with the solid support, as described further below. The solid support includes either the isolated HCV antigen, such as one or more NS3/4a conformational epitopes, or a MEFA as described above, the MEFA including one or more epitopes derived from the same region of the polyprotein as the

HCV antigen, such as from the NS3/4a region. As explained above, a number of antigens including such epitopes are known, including, but not limited to antigens derived from the c33c and c100 regions, as well as fusion proteins comprising an NS3 epitope, such as c25, and the antigenic determinant known as 5-1-1 which is partially within the NS4a region (see, Figure 1). These and other NS3/4a epitopes are useful in the present assays and are known in the art and described in, e.g., Houghton et al, U.S. Patent No. 5,350,671; Chien et al., *Proc. Natl. Acad. Sci. USA* (1992) 89:10011-10015; Chien et al., *J. Gastroent. Hepatol.* (1993) 8:S33-39; Chien et al., International Publication No. WO 93/00365; Chien, D.Y., International Publication No. WO 94/01778; and U.S. Patent Nos. 6,346,375 and 6,150,087, the disclosures of which are incorporated herein by reference in their entireties.

If the sample is infected with HCV, HCV antibodies to an epitope present on the solid support, will bind to the solid support components. A detectably labeled antigen that also reacts with the captured HCV antibody from the biological sample, is also added in the solution phase. For example, if the antigen bound to the solid support is an NS3/4a conformational epitope, the detectably labeled antigen used in the solution phase is a MEFA that includes an NS3/4a epitope. If the antigen bound to the solid support is a MEFA that includes an NS3/4a epitope, the detectably labeled antigen used in the solution phase includes a conformational NS3/4a epitope.

A representative assay under the invention is depicted in Figure 2. As shown in the figure, the solid support includes a conformational NS3/4a epitope. The biological sample is added to the solid support. HCV antibodies directed against the NS3/4a epitope present in the sample, will bind the NS3/4a conformational epitope on the solid support. Horse radish peroxidase (HRP)-labeled MEFA 12, including an epitope to which sample antibodies bind, is then added. MEFA 12 binds the antibody that is also bound by the NS3/4a conformational epitope. Unbound components are washed away and detection of the label indicates the presence of HCV infection.

The above-described antigen/antibody/antigen sandwich assays are particularly advantageous as the use of two antigens which bind sample antibody allows for the use of larger volumes of sample. Additionally, the assay can be completed quickly.

Production of Antigens for use in the HCV Immunoassays

As explained above, the molecules of the present invention are generally produced recombinantly. Thus, polynucleotides encoding HCV antigens for use with the present invention can be made using standard techniques of molecular biology. For example, polynucleotide sequences coding for the above-described molecules can be obtained using recombinant methods, such as by screening cDNA and genomic libraries from cells expressing the gene, or by deriving the gene from a vector known to include the same. Furthermore, the desired gene can be isolated directly from viral nucleic acid molecules, using techniques described in the art, such as in Houghton et al., U.S. Patent No. 5,350,671. The gene of interest can also be produced synthetically, rather than cloned. The molecules can be designed with appropriate codons for the particular sequence. The complete sequence is then assembled from overlapping oligonucleotides prepared by standard methods and assembled into a complete coding sequence. See, e.g., Edge (1981) *Nature* 292:756; Nambair et al. (1984) *Science* 223:1299; and Jay et al. (1984) *J. Biol. Chem.* 259:6311.

Thus, particular nucleotide sequences can be obtained from vectors harboring the desired sequences or synthesized completely or in part using various oligonucleotide synthesis techniques known in the art, such as site-directed mutagenesis and polymerase chain reaction (PCR) techniques where appropriate. See, e.g., Sambrook, *supra*. In particular, one method of obtaining nucleotide sequences encoding the desired sequences is by annealing complementary sets of overlapping synthetic oligonucleotides produced in a conventional, automated polynucleotide synthesizer, followed by ligation with an appropriate DNA ligase and amplification of the ligated nucleotide sequence via PVR. See, e.g., Jayaraman et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:4084-4088.

Additionally, oligonucleotide directed synthesis (Jones et al. (1986) *Nature* 54:75-82), oligonucleotide directed mutagenesis of pre-existing nucleotide regions (Riechmann et al. (1988) *Nature* 332:323-327 and Verhoeyen et al. (1988) *Science* 239:1534-1536), and enzymatic filling-in of gapped oligonucleotides using T_4 DNA polymerase (Queen et al. (1989) *Proc. Natl. Acad. Sci. USA* 86:10029-10033) can be used under the invention to provide molecules having altered or enhanced antigen-binding capabilities, and/or reduced immunogenicity.

Once coding sequences have been prepared or isolated, such sequences can be cloned into any suitable vector or replicon. Numerous cloning vectors are known to those of skill in the art, and the selection of an appropriate cloning vector is a matter of choice. Suitable vectors include, but are not limited to, plasmids, phages, transposons, cosmids, chromosomes or viruses which are capable of replication when associated with the proper control elements.

The coding sequence is then placed under the control of suitable control elements, depending on the system to be used for expression. Thus, the coding sequence can be placed under the control of a promoter, ribosome binding site (for bacterial expression) and, optionally, an operator, so that the DNA sequence of interest is transcribed into RNA by a suitable transformant. The coding sequence may or may not contain a signal peptide or leader sequence which can later be removed by the host in post-translational processing. See, e.g., U.S. Patent Nos. 4,431,739; 4,425,437; 4,338,397.

In addition to control sequences, it may be desirable to add regulatory sequences which allow for regulation of the expression of the sequences relative to the growth of the host cell. Regulatory sequences are known to those of skill in the art, and examples include those which cause the expression of a gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Other types of regulatory elements may also be present in the vector. For example, enhancer elements may be used herein to increase expression levels of the constructs. Examples

include the SV40 early gene enhancer (Dijkema et al. (1985) *EMBO J.* 4:761), the enhancer/promoter derived from the long terminal repeat (LTR) of the Rous Sarcoma Virus (Gorman et al. (1982) *Proc. Natl. Acad. Sci. USA* 79:6777) and elements derived from human CMV (Boshart et al. (1985) *Cell* 41:521), such as elements included in the CMV intron A sequence (U.S. Patent No. 5,688,688). The expression cassette may further include an origin of replication for autonomous replication in a suitable host cell, one or more selectable markers, one or more restriction sites, a potential for high copy number and a strong promoter.

An expression vector is constructed so that the particular coding sequence is located in the vector with the appropriate regulatory sequences, the positioning and orientation of the coding sequence with respect to the control sequences being such that the coding sequence is transcribed under the "control" of the control sequences (i.e., RNA polymerase which binds to the DNA molecule at the control sequences transcribes the coding sequence). Modification of the sequences encoding the molecule of interest may be desirable to achieve this end. For example, in some cases it may be necessary to modify the sequence so that it can be attached to the control sequences in the appropriate orientation; i.e., to maintain the reading frame. The control sequences and other regulatory sequences may be ligated to the coding sequence prior to insertion into a vector. Alternatively, the coding sequence can be cloned directly into an expression vector which already contains the control sequences and an appropriate restriction site.

As explained above, it may also be desirable to produce mutants or analogs of the antigen of interest. This is particularly true with NS3/4a. Methods for doing so are described in, e.g., Dasmahapatra et al., U.S. Patent No. 5,843,752 and Zhang et al., U.S. Patent No. 5,990,276. Mutants or analogs of this and other HCV proteins for use in the subject assays may be prepared by the deletion of a portion of the sequence encoding the polypeptide of interest, by insertion of a sequence, and/or by substitution of one or more nucleotides within the sequence. Techniques for modifying nucleotide sequences, such as site-directed mutagenesis, and the like, are well known to those skilled in the art. See,

e.g., Sambrook et al., supra; Kunkel, T.A. (1985) *Proc. Natl. Acad. Sci. USA* (1985) 82:448; Geisselsoder et al. (1987) *BioTechniques* 5:786; Zoller and Smith (1983) *Methods Enzymol.* 100:468; Dalbie-McFarland et al. (1982) *Proc. Natl. Acad. Sci USA* 79:6409.

The molecules can be expressed in a wide variety of systems, including insect, mammalian, bacterial, viral and yeast expression systems, all well known in the art.

For example, insect cell expression systems, such as baculovirus systems, are known to those of skill in the art and described in, e.g., Summers and Smith, *Texas Agricultural Experiment Station Bulletin No. 1555* (1987). Materials and methods for baculovirus/insect cell expression systems are commercially available in kit form from, *inter alia*, Invitrogen, San Diego CA ("MaxBac" kit). Similarly, bacterial and mammalian cell expression systems are well known in the art and described in, e.g., Sambrook et al., *supra*. Yeast expression systems are also known in the art and described in, e.g., *Yeast Genetic Engineering* (Barr et al., eds., 1989) Butterworths, London.

A number of appropriate host cells for use with the above systems are also known. For example, mammalian cell lines are known in the art and include immortalized cell lines available from the American Type Culture Collection (ATCC), such as, but not limited to, Chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS), human embryonic kidney cells, human hepatocellular carcinoma cells (e.g., Hep G2), Madin-Darby bovine kidney ("MDBK") cells, as well as others. Similarly, bacterial hosts such as *E. coli*, *Bacillus subtilis*, and *Streptococcus spp.*, will find use with the present expression constructs. Yeast hosts useful in the present invention include *inter alia*, *Saccharomyces cerevisiae*, *Candida albicans*, *Candida maltosa*, *Hansenula polymorpha*, *Kluyveromyces fragilis*, *Kluyveromyces lactis*, *Pichia guillerimondii*, *Pichia pastoris*, *Schizosaccharomyces pombe* and *Yarrowia lipolytica*. Insect cells for use with baculovirus expression vectors include, *inter alia*, *Aedes aegypti, Autographa californica, Bombyx mori, Drosophila*

melanogaster, Spodoptera frugiperda, and *Trichoplusia ni*.

Nucleic acid molecules comprising nucleotide sequences of interest can be stably integrated into a host cell genome or maintained on a stable episomal element in a suitable host cell using various gene delivery techniques well known in the art. *See, e.g.*, U.S. Patent No. 5,399,346.

Depending on the expression system and host selected, the molecules are produced by growing host cells transformed by an expression vector described above under conditions whereby the protein is expressed. The expressed protein is then isolated from the host cells and purified. If the expression system secretes the protein into growth media, the product can be purified directly from the media. If it is not secreted, it can be isolated from cell lysates. The selection of the appropriate growth conditions and recovery methods are within the skill of the art.

The recombinant production of various HCV antigens has been described. See, e.g., Houghton et al., U.S. Patent No. 5,350,671; Chien et al., *J. Gastroent. Hepatol.* (1993) 8:S33-39; Chien et al., International Publication No. WO 93/00365; Chien, D.Y., International Publication No. WO 94/01778.

Immunodiagnostic Assays

Once produced, the above HCV antigens are placed on an appropriate solid support for use in the subject immunoassays. A solid support, for the purposes of this invention, can be any material that is an insoluble matrix and can have a rigid or semi-rigid surface. Exemplary solid supports include, but are not limited to, substrates such as nitrocellulose (e.g., in membrane or microtiter well form); polyvinylchloride (e.g., sheets or microtiter wells); polystyrene latex (e.g., beads or microtiter plates); polyvinylidine fluoride; diazotized paper; nylon membranes; activated beads, magnetically responsive beads, and the like. Particular supports include plates, pellets, disks, capillaries, hollow fibers, needles, pins, solid fibers, cellulose beads, pore-glass beads, silica gels, polystyrene beads optionally cross-linked with divinylbenzene, grafted co-poly beads, polyacrylamide beads, latex beads, dimethylacrylamide beads optionally crosslinked with N-N'-bis-acryloylethylenediamine, and glass particles coated with a hydrophobic polymer.

If desired, the molecules to be added to the solid support can readily be functionalized to create styrene or acrylate moieties, thus enabling the incorporation of the molecules into polystyrene, polyacrylate or other polymers such as polyimide, polyacrylamide, polyethylene, polyvinyl, polydiacetylene, polyphenylene-vinylene, polypeptide, polysaccharide, polysulfone, polypyrrole, polyimidazole, polythiophene, polyether, epoxies, silica glass, silica gel, siloxane, polyphosphate, hydrogel, agarose, cellulose, and the like.

In one context, a solid support is first reacted with either the isolated HCV antigen or the MEFA (called "the solid-phase component" herein), under suitable binding conditions such that the molecules are sufficiently immobilized to the support. Sometimes, immobilization to the support can be enhanced by first coupling the antigen to a protein with better solid phase-binding properties. Suitable coupling proteins include, but are not limited to, macromolecules such as serum albumins including bovine serum albumin (BSA), keyhole limpet hemocyanin, immunoglobulin molecules,

thyroglobulin, ovalbumin, and other proteins well known to those skilled in the art. Other reagents that can be used to bind molecules to the support include polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, and the like. Such molecules and methods of coupling these molecules to antigens, are well known to those of ordinary skill in the art. See, e.g., Brinkley, M.A. (1992) *Bioconjugate Chem.* 3:2-13; Hashida et al. (1984) *J. Appl. Biochem.* 6:56-63; and Anjaneyulu and Staros (1987) *International J. of Peptide and Protein Res.* 30:117-124.

After reacting the solid support with the solid-phase components, any nonimmobilized solid-phase components are removed from the support by washing, and the support-bound components are then contacted with a biological sample suspected of containing HCV antibodies (called "ligand molecules" herein) under suitable binding conditions. After washing to remove any nonbound ligand molecules, a second HCV antigen (either an isolated HCV antigen or the MEFA, depending on which antigen is bound to the solid support), is added under suitable binding conditions. This second antigen is termed the "solution-phase component" herein. The added antigen includes a detectable label, as described above, and binds ligand molecules that have reacted with the support-bound antigen. Thus, the ligand molecules bind both the solid-phase component, as well as the solution-phase component. Unbound ligand molecules and solution-phase components are removed by washing. The presence of a label therefore indicates the presence of HCV antibodies in the biological sample.

More particularly, an ELISA method can be used, wherein the wells of a microtiter plate are coated with the solid-phase components. A biological sample containing or suspected of containing ligand molecules is then added to the coated wells. After a period of incubation sufficient to allow ligand-molecule binding to the immobilized solid-phase component, the plate(s) can be washed to remove unbound moieties and a detectably labeled solution-phase component is added. These molecules are allowed to react with any captured sample antibody, the plate washed and the presence of the label detected using methods well known in the art.

The above-described assay reagents, including the immunoassay solid support with bound antigens, as well as antigens to be reacted with the captured sample, can be provided in kits, with suitable instructions and other necessary reagents, in order to conduct immunoassays as described above. The kit can also contain, depending on the particular immunoassay used, suitable labels and other packaged reagents and materials (i.e. wash buffers and the like). Standard immunoassays, such as those described above, can be conducted using these kits.

III. Experimental

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperatures, etc.), but some experimental error and deviation should, of course, be allowed for.

EXAMPLE 1

Production of an NS3/4a Conformational Epitope with Thr to Pro and Ser to Ile Substitutions

A conformational epitope of NS3/4a was obtained as follows. This epitope has the sequence specified in Figures 3A through 3D (SEQ ID NOS:1 and 2) and differs from the native sequence at positions 403 (amino acid 1428 of the HCV-1 full-length sequence) and 404 (amino acid 1429 of the HCV-1 full-length sequence). Specifically, the Thr normally occurring at position 1428 of the native sequence has been mutated to Pro and Ser which occurs at position 1429 of the native sequence has been mutated to Ile.

In particular, the yeast expression vector used was pBS24.1. This yeast expression vector contains 2μ sequences and inverted repeats (IR) for autonomous replication in yeast, the α-factor terminator to ensure transcription termination, and the yeast *leu2-d* and URA3 for selection. The ColE1 origin of replication and the β-lactamase gene are also present for propagation and selection in *E. coli* (Pichuantes et al. (1996) "Expression of Heterologous Gene Products in Yeast." In: *Protein Engineering: A Guide to Design and Production*, Chapter 5. J. L. Cleland and C. Craik, eds., Wiley-Liss, Inc., New York, N.Y. pp. 129-161.

Plasmid pd.hcv1a.ns3ns4aPI, which encoded a representative NS3/4a epitope used in the subject immunoassays, was produced as follows. A two step procedure was used. First, the following DNA pieces were ligated together: (a) synthetic oligonucleotides which would provide a 5' *Hind*III cloning site, followed by the sequence ACAAAACAAA (SEQ ID NO: 8), the initiator ATG, and codons for HCV1a, beginning with amino acid 1027 and continuing to a *Bgl*I site at amino acid 1046; (b) a 683 bp *Bgl*I-*Cla*I restriction fragment (encoding amino acids 1046-1274) from pAcHLTns3ns4aPI; and (c) a pSP72 vector (Promega, Madison, WI, GenBank/EMBL Accession Number X65332) which had been digested with *Hind*III and *Cla*I,

dephosphorylated, and gel-purified. Plasmid pAcHLTns3ns4aPI was derived from pAcHLT, a baculovirus expression vector commercially available from BD Pharmingen (San Diego, CA). In particular, a pAcHLT *Eco*RI-*Pst*I vector was prepared, as well as the following fragments: *Eco*RI- *Alwn*I, 935 bp, corresponding to amino acids 1027-1336 of the HCV-1 genome; *Alwn*I-*Sac*II, 247 bp, corresponding to amino acids 1336-1419 of the HCV-1 genome; *Hinf*I-*Bgl*I, 175 bp, corresponding to amino acids 1449-1509 of the HCV-1 genome; *Bgl*I-*Pst*I, 619 bp, corresponding to amino acids 1510-1711 of the HCV-1 genome, plus the transcription termination codon. A *Sac*II -*Hinf*I synthetically generated fragment of 91 bp, corresponding to amino acids 1420-1448 of the HCV-1 genome and containing the PI mutations (Thr-1428 mutated to Pro, Ser-1429 mutated to Ile), was ligated with the 175 bp *Hinf*I-*Bgl*I fragment and the 619 bp *Bgl*I-*Pst*I fragment described above and subcloned into a pGEM-5Zf(+) vector digested with *Sac*II and *Pst*I. pGEM-5Zf(+) is a commercially available *E. coli* vector (Promega, Madison, WI, GenBank/EMBL Accession Number X65308). After transformation of competent HB101 cells, miniscreen analysis of individual clones and sequence verification, an 885 bp *Sac*II-*Pst*I fragment from pGEM5.PI clone2 was gel-purified. This fragment was ligated with the *Eco*RI-*Alwn*I 935 bp fragment, the *Alwn*I-*Sac*II 247 bp fragment and the pAcHLT *Eco*RI-*Pst*I vector, described above. The resultant construct was named pAcHLTns3ns4aPI.

The ligation mixture above was transformed into HB101-competent cells and plated on Luria agar plates containing 100 μg/ml ampicillin. Miniprep analyses of individual clones led to the identification of putative positives, two of which were amplified. The plasmid DNA for pSP72 1aHC, clones #1 and #2 were prepared with a Qiagen Maxiprep kit and were sequenced.

Next, the following fragments were ligated together: (a) a 761 bp *Hind*III-*Cla*I fragment from pSP721aHC #1 (pSP72.1aHC was generated by ligating together the following: pSP72 which had been digested with *Hind*III and *Cla*I, synthetic oligonucleotides which would provide a 5' *Hind*III cloning site, followed by the

sequence ACAAAACAAA (SEQ ID NO: 8), the initiation codon ATG, and codons for HCV1a, beginning with amino acid 1027 and continuing to a *Bgl*II site at amino acid 1046, and

a 683 bp *Bgl*II-*Cla*I restriction fragment (encoding amino acids 1046-1274) from pAcHLTns3ns4aPI); (b) a 1353 bp *Bam*HI-*Hind*III fragment for the yeast hybrid promoter ADH2/GAPDH; (c) a 1320 bp *Cla*I-*Sal*I fragment (encoding HCV1a amino acids 1046-1711 with Thr 1428 mutated to Pro and Ser 1429 mutated to Ile) from pAcHLTns3ns4aPI; and (d) the pBS24.1 yeast expression vector which had been digested with *Bam*HI and *Sal*I, dephosphorylated and gel-purified. The ligation mixture was transformed into competent HB101 and plated on Luria agar plates containing 100 μg/ml ampicillin. Miniprep analyses of individual colonies led to the identification of clones with the expected 3446 bp *Bam*HI-*Sal*I insert which was comprised of the ADH2/GAPDH promoter, the initiator codon ATG and HCV1a NS3/4a from amino acids 1027-1711 (shown as amino acids 1-686 of Figures 3A-3D), with Thr 1428 (amino acid position 403 of Figures 3A-3D) mutated to Pro and Ser 1429 (amino acid position 404 of Figures 3A-3D) mutated to Ile. The construct was named pd.HCV1a.ns3ns4aPI (see, Figure 4).

S. cerevisiae strain AD3 was transformed with pd.HCV1a.ns3ns4aPI and single transformants were checked for expression after depletion of glucose in the medium. The recombinant protein was expressed at high levels in yeast, as detected by Coomassie blue staining and confirmed by immunoblot analysis using a polyclonal antibody to the helicase domain of NS3.

EXAMPLE 2

Purification of NS3/4a Conformational Epitope

The NS3/4a conformational epitope was purified as follows. *S. cerevisiae* cells from above, expressing the NS3/4a epitope were harvested as described above. The cells were suspended in lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA,

1 mM PMSF, 0.1 μM pepstatin, 1 μM leupeptin) and lysed in a Dyno-Mill (Wab Willy A. Bachofon, Basel, Switzerland) or equivalent apparatus using glass beads, at a ratio of 1:1:1 cells:buffer:0.5 mm glass beads. The lysate was centrifuged at 30100 x g for 30 min at 4◦C and the pellet containing the insoluble protein fraction was added to wash buffer (6 ml/g start cell pellet weight) and rocked at room temperature for 15 min. The wash buffer consisted of 50 mM $NaPO_4$ pH 8.0, 0. 3 M NaCl, 5 mM β-mercaptoethanol, 10% glycerol, 0.05% octyl glucoside, 1 mM EDTA, 1 mM PMSF, 0.1 μM pepstatin, 1 μM leupeptin. Cell debris was removed by centrifugation at 30100 x g for 30 min at 4◦C. The supernatant was discarded and the pellet retained.

Protein was extracted from the pellet as follows. 6 ml/g extraction buffer was added and rocked at room temperature for 15 min. The extraction buffer consisted of 50 mM Tris pH 8.0, 1 M NaCl, 5 mM β-mercaptoethanol, 10% glycerol, 1 mM EDTA, 1 mM PMSF, 0.1 μM pepstatin, 1 μM leupeptin. This was centrifuged at 30100 x g for 30 min at 4◦C. The supernatant was retained and ammonium sulfate added to 17.5% using the following formula: volume of supernatant (ml) multiplied by x% ammonium sulfate/(1 - x% ammonium sulfate) = ml of 4.1 M saturated ammonium sulfate to add to the supernatant. The ammonium sulfate was added dropwise while stirring on ice and the solution stirred on ice for 10 min. The solution was centrifuged at 17700 x g for 30 min at 4◦C and the pellet retained and stored at 2◦C to 8◦C for up to 48 hrs.

The pellet was resuspended and run on a Poly U column (Poly U Sepharose 4B, Amersham Pharmacia) at 4◦C as follows. Pellet was resuspended in 6 ml Poly U equilibration buffer per gram of pellet weight. The equilibration buffer consisted of 25 mM HEPES pH 8.0, 200 mM NaCl, 5 mM DTT (added fresh), 10% glycerol, 1.2 octyl glucoside. The solution was rocked at 4□C for 15 min and centrifuged at 31000 x g for 30 min at 4◦C.

A Poly U column (1 ml resin per gram start pellet weight) was prepared. Linear flow rate was 60 cm/hr and packing flow rate was 133% of 60 cm/hr. The column was equilibrated with equilibration buffer and the supernatant of the resuspended ammonium

sulfate pellet was loaded onto the equilibrated column. The column was washed to baseline with the equilibration buffer and protein eluted with a step elution in the following Poly U elution buffer: 25 mM HEPES pH 8.0, 1 M NaCl, 5 mM DTT (added fresh), 10% glycerol, 1.2 octyl glucoside. Column eluate was run on SDS-PAGE (Coomassie stained) and aliquots frozen and stored at -80°C. The presence of the NS3/4a epitope was confirmed by Western blot, using a polyclonal antibody directed against the NS3 protease domain and a monoclonal antibody against the 5-1-1 epitope (HCV 4a).

Additionally, protease enzyme activity was monitored during purification as follows. An NS4A peptide (KKGSVVIVGRIVLSGKPAIIPKK) (SEQ ID NO: 9), and the sample containing the NS3/4a conformational epitope, were diluted in 90 µl of reaction buffer (25 mM Tris, pH 7.5, 0.15M NaCl, 0.5 mM EDTA, 10% glycerol, 0.05 n-Dodecyl B-D-Maltoside, 5 mM DTT) and allowed to mix for 30 minutes at room temperature. 90 µl of the mixture were added to a microtiter plate (Costar, Inc., Corning, NY) and 10 µl of HCV substrate (AnaSpec, Inc., San Jose CA) was added. The plate was mixed and read on a Fluostar plate reader. Results were expressed as relative fluorescence units (RFU) per minute.

Using these methods, the product of the 1 M NaCl extraction contained 3.7 RFU/min activity, the ammonium sulfate precipitate had an activity of 7.5 RFU/min and the product of the Poly U purification had an activity of 18.5 RFU/min.

EXAMPLE 3

Immunoreactivity of NS3/4a Conformational Epitope Verus Denatured NS3/4a

The immunoreactivity of the NS3/4a conformational epitope, produced as described above, was compared to NS3/4a which had been denatured by adding SDS to the NS3/4a conformational epitope preparation to a final concentration of 2%. The denatured NS3/4a and conformational NS3/4a were coated onto microtiter plates as described above. The c200 antigen (*Hepatology* (1992) 15:19-25, available in the

ORTHO HCV Version 3.0 ELISA Test System, Ortho-Clinical Diagnostics, Raritan, New Jersey) was also coated onto microtiter plates. The c200 antigen was used as a comparison it is presumed to be non-conformational due to the presence of reducing agent (DTT) and detergent (SDS) in its formulation.

The immunoreactivity was tested against two early HCV seroconversion panels, PHV 904 and PHV 914 (commercially available human blood samples from Boston Biomedica, Inc., West Bridgewater, MA). The results are shown in Table 6. The data suggest that the denatured or linearized form of NS3/4a (as well as c200) does not detect early seroconversion panels as early as the NS3/4a conformational epitope.

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TABLE 6

	NS3/4a vs. denatured NS3/4a							
	*Spiked 2% SDS to stock NS3/4a							
		NS3/4a	dNS3/4a*	c200		NS3/4a	dNS3/4a*	c200
		OD	OD	OD		s/co	s/co	s/co
HCV	PHV 904-1	0.012	0.012	0.009		0.02	0.02	0.01
Seroconversions	PHV 904-2	0.011	0.009	0.008		0.02	0.01	0.01
	PHV 904-3	1.124	0.071	0.045		1.80	0.11	0.07
	PHV 904-4	2.401	0.273	0.129		3.85	0.44	0.21
	PHV 904-5	3.022	0.793	0.347		4.85	1.28	0.57
	PHV 904-6	2.711	1.472	0.774		4.35	2.37	1.28
	PHV 904-7	3.294	1.860	0.943		5.28	2.99	1.55
	PHV 914-1	0.006	0.004	0.001		0.01	0.01	0.00
	PHV 914-2	0.005	0.004	0.002		0.01	0.01	0.00
	PHV 914-3	0.098	0.003	0.001		0.16	0.00	0.00
	PHV 914-4	1.118	0.006	0.004		1.79	0.01	0.01
	PHV 914-5	2.035	0.044	0.022		3.26	0.07	0.04
	PHV 914-6	2.092	0.074	0.025		3.35	0.12	0.04
	PHV 914-7	2.519	0.281	0.132		4.04	0.45	0.22
	PHV 914-8	2.746	0.907	0.500		4.40	1.46	0.82
	PHV 914-9	3.084	1.730	0.931		4.94	2.78	1.53
HCV 3.0	Neg. Cont.	0.023	0.024	0.008				
Controls	Neg. Cont.	0.027	0.024	0.007				
	Neg. Cont.	0.021	0.017	0.005				
	average	0.024	0.022	0.007				
	cutoff	0.624	0.622	0.607				
	Pos. Cont.	1.239	0.903	0.575		1.99	1.45	0.95
	Pos. Cont.	1.445	0.916	0.614		2.32	1.47	1.01

Immunoreactivity of the conformational epitope was also tested using monoclonal antibodies to NS3/4a, made using standard procedures. These monoclonal antibodies were then tested in the ELISA format against NS3/4a and denatured NS3/4a and c200 antigen. The data show that anti-NS3/4a monoclonals react to the NS3/4a and denatured NS3/4a in a similar manner to the seroconversion panels shown in Table 7. This result also provides further evidence that the NS3/4a is conformational in nature as monoclonal antibodies can be made which are similar in reactivity to the early c33c seroconversion panels.

Table 7				
		Plate		
		NS3/4a	dNS3/4a	c200
Monoclonal		OD	OD	OD
4B9/E3	1:100	1.820	0.616	0.369
	1:1000	1.397	0.380	0.246
	1:10000	0.864	0.173	0.070
	1:20000	0.607	0.116	0.085
5B7/D7	1:100	2.885	0.898	0.436
	1:1000	2.866	0.541	0.267
	1:10000	1.672	0.215	0.086
	1:20000	1.053	0.124	0.059
1A8/H2	1:100	1.020	0.169	0.080
	1:1000	0.921	0.101	0.043
	1:10000	0.653	0.037	0.013
	1:20000	0.337	0.027	0.011

EXAMPLE 4

Coating Solid Support with the HCV Antigens

The HCV NS3/4a conformational epitope or MEFA antigen is coated onto plates as follows. HCV coating buffer (50 mM NaPO4 pH 7.0, 2 mM EDTA and 0.1% Chloroacetamide) is filtered through a 0.22 μ filter unit. The following reagents are then added sequentially to the HCV coating buffer and stirred after each addition: 2 μg/ml BSA-Sulfhydryl Modified, from a 10 mg/ml solution (Bayer Corp. Pentex, Kankakee, Illinois); 5 mM DTT from a 1 M solution (Sigma, St. Louis, MO); 0.45 μg/ml NS3/4a (protein concentration of 0.3 mg/ml); or 0.375 μg/ml MEFA 7.1 (protein concentration of 1 mg/ml). The final solution is stirred for 15 minutes at room temperature.

200 μl of the above solution is added to each well of a Costar high binding, flat bottom plate (Corning Inc., Corning, New York) and the plates are incubated overnight in a moisture chamber. The plates are then washed with wash buffer (1 x PBS, 0.1% TWEEN-20), Tapped dry and 285 μl Ortho Post-Coat Buffer (1 x PBS, pH 7.4, 1% BSA, 3% sucrose) added. The plates are incubated for at least 1 hour, tapped and dried overnight at 2-8°C. The plates are pouched with desiccants for future use.

EXAMPLE 5

Immunoassays

In order to test the ability of the subject immunoassays to detect HCV infection, panels of commercially available human blood samples are used which are HCV-infected. Such panels are commercially available from Boston Biomedica, Inc., West Bridgewater, MA (BBI); Bioclinical Partners, Franklin, MA (BCP); and North American Biologics, Inc., BocoRatan, FL (NABI).

The assay is conducted as follows. 200 μl of specimen diluent buffer (1 g/l casein, 100 mg/l recombinant human SOD, 1 g/l chloracetamide, 10 g/l BSA, 500 mg/l yeast extract, 0.366 g/l EDTA, 1.162 g/l KPO_4, 5 ml/l Tween-20, 29.22 g/l NaCl, 1.627

g/l $NaPO_4$, 1% SDS) is added to the coated plates. 20 µl of sample is then added. This is incubated at 37°C for 30-60 minutes. The plates are washed with wash buffer (1 x PBS, pH 7.4, 0.1% Tween-20). 200 µl of the labeled solution-phase component (either HRP-labeled MEFA or HRP-labeled NS3/4a conformational epitope, depending on the antigen bound to the solid support), diluted 1:22,000 in ORTHO HCV 3.0 ELISA Test System with Enhanced SAVe bulk conjugate diluent (Ortho-Clinical Diagnostics, Raritan, New Jersey) is added and incubated for 30-60 minutes at 37□C. This is washed as above, and 200 µl substrate solution (1 OPD tablet/10ml) is added. The OPD tablet contains *o*-phenylenediamine dihydrochloride and hydrogen peroxide for horse radish peroxidase reaction color development. This is incubated for 30 minutes at room temperature in the dark. The reaction is stopped by addition of 50 µl 4N H_2SO_4 and the plates are read at 492 nm, relative to absorbance at 690 nm as control.

Accordingly, novel HCV detection assays have been disclosed. From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the disclosure herein.